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Pathogenicity of *Salmonella* Strains Isolated from Egg Shells and the Layer Farm Environment in Australia

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In Australia, the egg industry is periodically implicated during outbreaks of *Salmonella* food poisoning. *Salmonella enterica* serovar Typhimurium and other nontyphoidal *Salmonella* spp., in particular, are a major concern for Australian public health. Several definitive types of *Salmonella* Typhimurium strains, but primarily *Salmonella* Typhimurium definitive type 9 (DT9), have been frequently reported during egg-related food poisoning outbreaks in Australia. The aim of the present study was to generate a pathogenicity profile of nontyphoidal *Salmonella* isolates obtained from Australian egg farms. To achieve this, we assessed the capacity of *Salmonella* isolates to cause gastrointestinal disease using both *in vitro* and *in vivo* model systems. Data from *in vitro* experiments demonstrated that the invasion capacity of *Salmonella* serovars cultured to stationary phase (liquid phase) in LB medium was between 90- and 300-fold higher than bacterial suspensions in normal saline (cultured in solid phase). During the *in vivo* infection trial, clinical signs of infection and mortality were observed only for mice infected with either 10^3 or 10^5 CFU of *S.* Typhimurium DT9. No mortality was observed for mice infected with *Salmonella* serovars with medium or low invasive capacity in Caco-2 cells. Pathogenicity gene profiles were also generated for all serovars included in this study. The majority of serovars tested were positive for selected virulence genes. No relationship between the presence or absence of virulence genes by PCR and either *in vitro* invasive capacity or *in vivo* pathogenicity was detected. Our data expand the knowledge of strain-to-strain variation in the pathogenicity of Australian egg industry-related *Salmonella* spp.

Salmonella is one of the most common causes of food-borne infection worldwide. In Australia, the egg industry is periodically implicated in cases of *Salmonella* food poisoning (1). Uncooked or partially cooked foods containing raw egg as an ingredient accounted for 14% of food-borne outbreaks in 2006, 13% in 2007, and 28% in the first quarter of 2008 (2). It has been shown that some *Salmonella* serovars, such as *Salmonella enterica* serovar Enteritidis, have the capacity to infect developing eggs within the oviduct, and therefore contaminated eggs act as an ecological amplifier (3). It is believed that this could then facilitate the dissemination of *Salmonella* into the food chain and its eventual transmission to humans. These studies, however, for the most part have been focused on *Salmonella* Enteritidis with limited investigation of *S. enterica* serovar Typhimurium. The dramatic increase in *Salmonella* Enteritidis infections occurring in overseas countries has not been observed in Australia (4). *Salmonella* Typhimurium and other nontyphoidal *Salmonella* spp., however, have become a major concern for the Australian egg industry.

S. enterica serovars are a diverse group of pathogens that have evolved to survive in a wide range of environments and across multiple hosts (5). There are several definitive types of *Salmonella* Typhimurium but *Salmonella* Typhimurium definitive type 9 (DT9) has been frequently reported from egg-related food poisoning outbreaks in Australia (6). Other nontyphoidal serovars such as *S. enterica* Infantis, *S. enterica* Oranienburg, and *S. enterica* Lille are also frequently isolated from egg-layer farms or eggs (6); however, these serovars have not been reported in egg-related food poisoning cases in Australia. In humans, *Salmonella* Typhimurium can cause self-limiting gastroenteritis and has been found to be the most commonly reported serovar in both North America and Oceania (7). Septicemia associated with nontyphoidal *Salmonella* is also a growing public health concern, which can affect healthy as well as certain populations of immunodeficient individuals (8).

Complex pathogenesis is characteristic of *Salmonella* infection. The virulence of *Salmonella* is encoded by multiple genes which are clustered on *Salmonella* pathogenicity islands (SPIs) (9). SPIs have the potential to contribute to the overall pathogenesis of the bacterium (9). Genomic variability among bacterial strains arises primarily as a consequence of horizontal gene transfer (10). This inherent variability is likely the source of the various pathogenicities among nontyphoidal *Salmonella* strains. Consequently, characterization of the virulence gene repertoire by PCR has been used by many groups to profile the virulence of *Salmonella* (11, 12).

In Australia, there is limited current information regarding the characterization of the overall virulence of nontyphoidal *Salmonella* strains isolated from eggs or the egg farm environment. Our hypothesis is that *Salmonella* serovars recovered specifically from layer farm environments or egg shell wash possess various levels of pathogenicity. Other studies that have characterized the pathogenic potential of nontyphoidal *Salmonella* strains have focused mainly on *Salmonella* Enteritidis isolates recovered from either

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TABLE 1 *Salmonella* serovars isolated from Australian egg shell wash and egg farm environment[a]

S. enterica serovar[b]	Source of isolate	No. of isolates from egg farms by year			
		2010	2011	2012	2013
Typhimurium DT9	Egg shell wash	17	69	17	50
Infantis	Egg shell wash	27	122	9	7
Agona	Egg belt	18	85	25	4
Mbandaka	Egg belt	11	10	1	129
Johannesburg	Feces	0	3	0	0
Livingstone	Feces	2	0	0	0
Subspecies I 4,12:d:−	Feces	6	8	1	4
Chester	Feces	3	1	0	3
Zanzibar	Feces	0	5	0	0
Kiambu	Feces	7	19	0	0
Lille	Feces	3	4	0	0
Ohio	Feces	17	25	6	0
Anatum	Feces	16	46	0	13
Havana	Feces	0	26	0	0
Oranienburg	Feces	0	0	12	73
Montevideo	Feces	12	49	1	0
Orion var. 15^+,34^+	Dust in layer shed	10	28	0	0

[a] Information was obtained from the *Salmonella* Reference Laboratory, Australian *Salmonella* Reference Centre, SA Pathology, Adelaide, Australia (37–40).
[b] Serovars used in the present study.

human clinical cases or non-poultry-associated environmental sources (13, 14).

Previously, *Salmonella* pathogenicity has been characterized using an *in vitro* invasion assay utilizing the human colon tumorigenic cell line Caco-2 (15). Major studies investigating the pathogenicity of *Salmonella* serovars using a human epithelial cell model system have shown variation in the levels of pathogenicity among different serovars (10). Invasion in cultured epithelial cells is routinely used as a measure of pathogenicity of *Salmonella* isolates (12). To date, no studies have been conducted to characterize the pathogenicity of field isolates of *Salmonella* obtained from egg farms in Australia.

The central aim of the present study was to generate an overall pathogenicity profile of multiple nontyphoidal *Salmonella* isolates sourced from Australian egg farms. To achieve this, the capacity of these isolates to cause gastrointestinal and invasive disease was investigated using both *in vitro* and *in vivo* models.

MATERIALS AND METHODS

***Salmonella* isolates.** All *Salmonella* serovars were obtained from the *Salmonella* Reference Laboratory, Institute of Veterinary Medical Science (IMVS), Adelaide, South Australia. The cultures were collected on xylose lysine deoxycholate (XLD) agar (Oxoid, Australia) and incubated overnight at 37°C. Long-term stocks were generated by preparing a bacterial suspension in brain heart infusion broth containing 20% glycerol and storing the stocks at −80°C. The list of cultures is provided in Table 1. The serovars selected in this study are commonly isolated from egg farms or egg shell wash (Table 1).

DNA extraction and PCR. Overnight cultures of the *Salmonella* serovars selected for this study were grown at 37°C in 4 ml of Luria-Bertani (LB) broth. DNA was purified from 1 ml of bacteria using a Promega genomic DNA kit (Promega, USA). Purified DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Australia). Working DNA solutions were prepared by diluting stock solutions to 5 ng/μl. Primers for each gene were designed using the primer design feature in GenBank or obtained from Hughes et al. (16). Primers were obtained from GeneWorks (Adelaide, South Australia) (Table 2). PCR mix was comprised of 1× *Taq* polymerase buffer (Fisher Biotec, Australia), 1.0 mM $MgCl_2$, 0.5 μM each forward and reverse primer, 0.2 μM each deoxynucleoside triphosphate (dNTP), 0.3 units of *Taq* polymerase (Fisher Biotec, Australia), and 10 ng of *Salmonella* DNA.

Product sizes for each of the genes were relatively similar; therefore, PCR conditions were nearly universal. The primer annealing temperatures were the only variation during PCR, and these are listed in the Table 2. The general reaction protocol was as follows: 95°C for 5 min (step 1); 30 cycles (step 2) of 95°C for 30 s (melt), 45 s at the annealing temperature specified in Table 2, and 72°C for 1 min (extension); 72°C for 4 min (step 3); and a final hold at 8°C (step 4).

Tissue culture. A human colonic carcinoma cell line, Caco-2, was obtained from the American Tissue Culture Collection (ATCC). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Australia) supplemented with 10% fetal bovine serum (FBS) (HyClone, Australia) containing 100 U/ml penicillin and 100 μg/ml streptomycin (HyClone, Australia). Caco-2 cells were expanded and maintained as frozen stocks. Cell line stocks tested negative for mycoplasma. Cells were used between passages 4 and 9 for gentamicin protection invasion assays.

Gentamicin protection *in vitro* invasion assay. The gentamicin protection assay was performed using Caco-2 cells to characterize the capacity of the selected *Salmonella* serotypes for invasion into epithelial tissue. Polarized monolayers of Caco-2 cells were obtained by seeding cells into each well of a 48-well tray. The progression to polarization was monitored by measuring alkaline phosphatase production. When alkaline phosphatase levels plateaued (after 12 to 13 days), cells were considered to be polarized and were used within 24 h. Cell culture medium was changed every other day during polarization. All invasion assays were performed in replicate and repeated five times.

The gentamicin MIC was determined for all serovars included in this study using the Clinical and Laboratory Standards Institute (CLSI) protocol (17). The gentamicin MIC for all serovars was less than 0.25 μg/ml. For invasion assays, 400 μg/ml gentamicin was used to eliminate bacteria that had not invaded (18).

For the invasion assay experiments, bacteria were plated fresh 24 h prior to the experiments. Suspensions were prepared in normal saline by selecting individual colonies from agar plates. The optical densities at 600 nm (OD_{600}) of the suspensions for each serovar were measured and adjusted to between 0.150 and 0.200, corresponding to 10^8 CFU/ml. An inoculum check was performed by doing a dilution series of the stock on nutrient agar plates.

Invasion assays were also performed using bacteria prepared in LB suspensions. These were prepared by selecting a single colony and placing it into 3 ml of LB broth. Tubes were incubated at 37°C for 6 h with shaking at 50 rpm. Ten microliters of this suspension was then added to 4 ml of LB medium and incubated overnight at 37°C with shaking at 50 rpm. This method yielded bacterial cultures that were in a stationary (liquid) phase of growth.

To investigate the difference in invasion abilities of stationary and non-stationary-phase cells (overnight cultures in solid phase versus liquid phase) of *Salmonella* spp., the invasion assays were performed using bacteria suspended in normal saline and LB medium. On the day of the invasion assay, Caco-2 cells were lifted from a single well from each of two 48-well trays and counted using a hemocytometer. An average number of cells was obtained and used to calculate the multiplicity of infection (MOI). An MOI of 100 was selected to statistically distinguish between low-invasive, moderately invasive, and highly invasive *Salmonella* serovars.

Prior to the addition of bacteria, Caco-2 cells were washed three times with DMEM containing no supplements to remove any residual culture medium antibiotics or FBS. The final DMEM wash was replaced with 500 μl of fresh DMEM. Bacteria were added to individual wells. Experiments

TABLE 2 Details of the genes, their role, primer sequence and targeted amplicon size used for *Salmonella* typing

Virulence gene	Function	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temp (°C)	Reference or source
prgH	Invasion protein	GCCCGAGCAGCCTGAGAAGTTAGAAA	TGAAATGAGCGCCCCTTGAGCCAGTC	55	32
sopB	Invasion and intracellular replication	GAAGACTACCAGGCGCACTT	TTGTGGATGTCCACGGTGAG	55	32
invA	Invasion protein	CTGGCGGTGGGTTTTGTTGTCTTCTCTATT	AGTTTCTCCCCCTCTTCATGCGTTACCC	60	32
sitC	Iron transporter	CAGTATATGCTCAACGCGATGTGGGTCTCC	CGGGGCGAAAATAAAGGCTGTGATGAAC	64	32
spiC	Disruption of Golgi apparatus and lysosomes	CCTGGATAATGACTATTGAT	AGTTTATGGTGATTGCGTAT	56	32
sifA	Required for maintenance of SCV[a]	TTTGCCGAACGCGCCCCCACACG	GTTGCCTTTTCTTGCGCTTTCCACCCATCT	62	32
misL	Adhesin	GTCGGCGAATGCCGCGAATA	GCGCTGTTAACGCTAATAGT	58	32
orfL	Survival within macrophages	GGAGTATCGATAAAGATGTT	GCGCGTAACGTCAGAATCAA	56	32
pipD	Colonization	CGGCGATTCATGACTTTGAT	CGTTATCATTCGGATCGTAA	58	32
iroN	Iron transport	ACTGGCACGGCTCGCTGTCGCTCTAT	CGCTTTACCGCCGTTCTGCCACTGC	60	32
pefA	Plasmid encoded fimbriae	GCGCCGCTCAGCCGAACCAG	CAGCAGAAGCCCAGGAAACAGTG	58	33
spvC	Modulation of host immune response	TCTCTGCATTTCGCCACCAT	TGCACAACCAAATGCGGAAG	58	33
sipA	Invasion protein	TACCCCTGCTGCTACGTAAT	CTCCAGGGCTTTACGTATCA	60	33
sipB	Invasion protein	TGGCAGGCGATGATTGAGTC	CCCATAATGCGGTTCGTTTC	58	This study
sipC	Invasion protein	TGCCCTGGCAAATAATGTCA	CATCGATTCGGGTCATATCC	58	33
fliC	Flagella protein	TACGCTGAATGTGCAACAAA	TACCGTCATCTGCAGTGTAT	58	This study
sopA	Induction of proinflammatory response	GCCCACGGTTTCTGAAGGTA	AAAGAGTCCGCTGTGAGTGG	60	This study
sipD	Invasion protein	TGCGTCAGCGTCTGTAATGT	GGCCTTATTTAGCGCTTCGC	58	This study
avrA	Modulation of host immune response	ATACTGCTTCCCGCCGC	ACACCGAAGCATTGACCTGT	58	This study
sptP	Disruption of actin cytoskeleton	TTCACCCTATCCGCCAGGTA	GTGTAGCCCGGTTCTCACAA	58	16
hilA	Activates expression of invasion genes	CACCAACCCGCTTCTCTCTT	ATTGTGGTCCAGCTCTGTCG	58	35
xthA	Survival in egg	CGAAAAACACCAGCCCGATG	CCGGCAGGAAGGAGCATTTA	55	36
yafD	Survival in egg	CGGATCCGTATCCTCGTGTG	ATCGTCAGTGAAACGCACCT	55	36
stn	Enterotoxin	CTTTGGTCGTAAAATAAGGCG	TGCCCAAAGCAGAGAGATTC	55	32

[a] SCV, *Salmonella*-containing vacuoles.

TABLE 3 Treatment groups for *Salmonella* infection trial[a]

Salmonella serovar	Invasiveness level	Invasiveness group
Typhimurium DT 9	High	C
Infantis	Low	A
Lille	Low	A
Oranienburg	Moderate	B
Montevideo	Moderate	B
Typhimurium ATCC 14028 (positive control)	High	C

[a] Animals received doses of either 10^3 CFU or 10^5 CFU of *Salmonella* ($n = 7$ animals/treatment for each serovar).

were performed in replicate. Following the addition of *Salmonella*, Caco-2 cells were incubated with bacteria for 2 h at 37°C and 5% CO_2. Cells were then washed three times with DMEM with no supplements. To kill any adherent bacteria, 400 μg/ml gentamicin diluted in DMEM was added to the Caco-2 cells. Cells were incubated in gentamicin for 15 min at 37°C and 5% CO_2. Cells were then washed three times with calcium- and magnesium-free phosphate-buffered saline (PBS). Finally, 1 ml of sterile 1% Triton X-100 was added to each well to lyse the cells and collect the intracellular bacteria. A serial 10-fold dilution series was prepared from the 1 ml of bacteria/cell lysate. One hundred microliters of each dilution was plated onto nutrient agar plates. Plates were incubated at 37°C overnight, and colonies were counted for each dilution of each serovar.

Mouse infection trial with selected isolates. The BALB/c mouse strain was selected for these studies. Mouse challenge studies were performed according to animal ethics protocol approved by the University of Adelaide (UA) Animal Care and Use Committee. Six- to 8-week-old, specific-pathogen-free, female mice weighing between 10 and 14 g were obtained from Laboratory Animal Services (University of Adelaide). Mice were raised in isolator cages and fed on a *Salmonella*-free commercial diet. Food and water were supplied *ad libitum* until the end of the experiment.

Salmonella serovars with different cell invasion potentials (as determined from data obtained from *in vitro* experiments) were used for *in vivo* experiments (Table 3). Along with *Salmonella* Typhimurium DT9, other nontyphoidal *Salmonella* spp., such as *Salmonella* Infantis, *S. enterica* serovar Montevideo, and *Salmonella* Oranienburg, were also isolated from human cases.

Bacterial isolates were grown overnight in Luria-Bertani (LB) broth and serially diluted in PBS to obtain desired cell counts. Mice from each treatment group were inoculated by oral gavage with either 10^3 or 10^5 CFU/ml of LB containing *Salmonella* culture. Control mice were inoculated with sterile LB broth. Each treatment group contained seven animals. To exclude observer bias in the interpretation of results, all clinical and bacteriological assessments were conducted by personnel blinded to the identity of the challenge isolates.

Challenged mice were observed at least twice daily for mortality and clinical parameters of disease, including ruffled fur, hunching behavior, and lethargy, for up to 21 days postinfection (p.i.). Mice showing all of the above symptoms were considered moribund and were euthanized by carbon dioxide asphyxiation. An experiment was terminated at day 21 p.i. when all the surviving mice were euthanized. The ATCC *Salmonella* Typhimurium strain 14028 was included as a positive control. The negative-control was LB broth.

***Salmonella* isolation from fecal samples by culture method.** Fecal pellets were collected from each mouse at 3, 6, 9, 12, 15, and 18 days postinfection (p.i.) and processed for isolation of challenge isolates by culture (fecal enrichment method) according to a previously described method (4, 19).

DNA extraction from fecal samples for real-time PCR. DNA from feces was extracted using a QIAamp DNA stool minikit (Qiagen, Australia) as per the manufacturer's guidelines. Fecal samples (0.2 g) collected from 0 to 18 days postinfection (p.i.) from each treatment group were weighed and treated with 2 ml of QIAamp stool lysis buffer. The samples were centrifuged at 4,800 × *g* for 10 min, and 120 μl of the supernatant was treated with Inhibitex (tablet; Qiagen, Australia). The samples were then centrifuged, and the supernatant was treated with proteinase K and lysis buffer. Washing and elution were performed using a spin column according to the manufacturer's instructions. Extracted DNA was quantified using a spectrophotometer (NanoDrop ND 1000) and stored at −70°C until used for real-time PCR (RT-PCR). Five nanograms of fecal DNA was used for the real-time PCR.

Quantitative PCR (qPCR). *Salmonella* shedding in fecal material was quantified using real-time PCR (RT-PCR). RT-PCR was performed using a Rotor Gene 3000 real-time PCR machine (Qiagen, Australia) and a TaqMan *Salmonella enterica* detection kit (Applied Biosystems, Australia). Each reaction mixture contained 9 μl of qPCR supermix and 6 μl of DNA template (12 ng) in a total reaction volume of 15 μl. The cycling parameters were 95°C for 10 min and 40 cycles at 95°C for 15 s, followed by 60°C for 60 s. All real-time PCR runs included negative and positive controls. The data were analyzed by two-way analysis of variance (ANOVA).

A standard curve was generated by preparing a serial dilution of a *Salmonella* strain. Bacteria were resuscitated on XLD agar overnight at 37°C. The individual isolated colonies were then suspended in 2 ml of PBS and matched with a 0.5 McFarland standard (bioMérieux, Australia). Serial dilutions were performed to achieve 10^8 CFU/ml. The CFU counts were confirmed by spreading serial dilutions on XLD agar plates. In order to determine the limit of detection of qPCR, fecal samples were spiked with various concentrations (10^8 CFU/ml to 10^0 CFU/ml) of *Salmonella*. qPCR was performed on serial dilutions (10^8 to 10^0) of genomic DNA, and a proportionality relationship was produced by plotting the threshold cycle (C_T) value against the logarithm of the CFU number. *Salmonella* copies were calculated using a standard curve prepared by serial 10-fold dilution of a cultured *Salmonella* sp.

Statistical analysis. The data were normally distributed. One-way analysis of variance (ANOVA) was used with Tukey's correction for multiple comparisons. Kaplan-Meier survival curves were generated for the *in vivo* mouse infection experiment. A log rank Mantel-Cox test was used to compare survival curves. A *k*-means cluster analysis was performed to identify invasive group types. All tests were run using either SPSS, version 21, or GraphPad Prism, version 6.0.

RESULTS AND DISCUSSION

The *Salmonella* genus is comprised of two species, *S. enterica* and *S. bongori*. Members of both species possess the ability to infect host cells while members of *S. enterica* are able to cause systemic infection (20). *S. enterica* is highly diverse, containing over 2,500 different serovars. Representative serovars from this species are the most commonly isolated serovars during outbreaks of foodborne salmonellosis, including *S.* Enteritidis, *S.* Typhimurium, *S. enterica* serovar Virchow, and *S.* Infantis (21). Many other *S. enterica* serotypes, however, are commonly isolated from eggs and egg-related products as well as from the layer farm environment, yet their degree of pathogenicity remains largely uncharacterized. In the present study, we have examined three parameters, *in vitro* cell invasion, virulence gene profile, and *in vivo* pathogenicity, of 17 *Salmonella* serovars isolated directly from egg shell wash or the egg farm environment.

***In vitro* intestinal epithelial invasion.** Surface contamination of an egg shell with *Salmonella* has been reported to be as high as 10^6 CFU (19). Thus, improper egg handling or storage conditions can lead to kitchen contamination or direct exposure of an individual to bacteria. Bacteria on the surface of the egg shell would be in a nutrient-poor environment and as such would not be express-

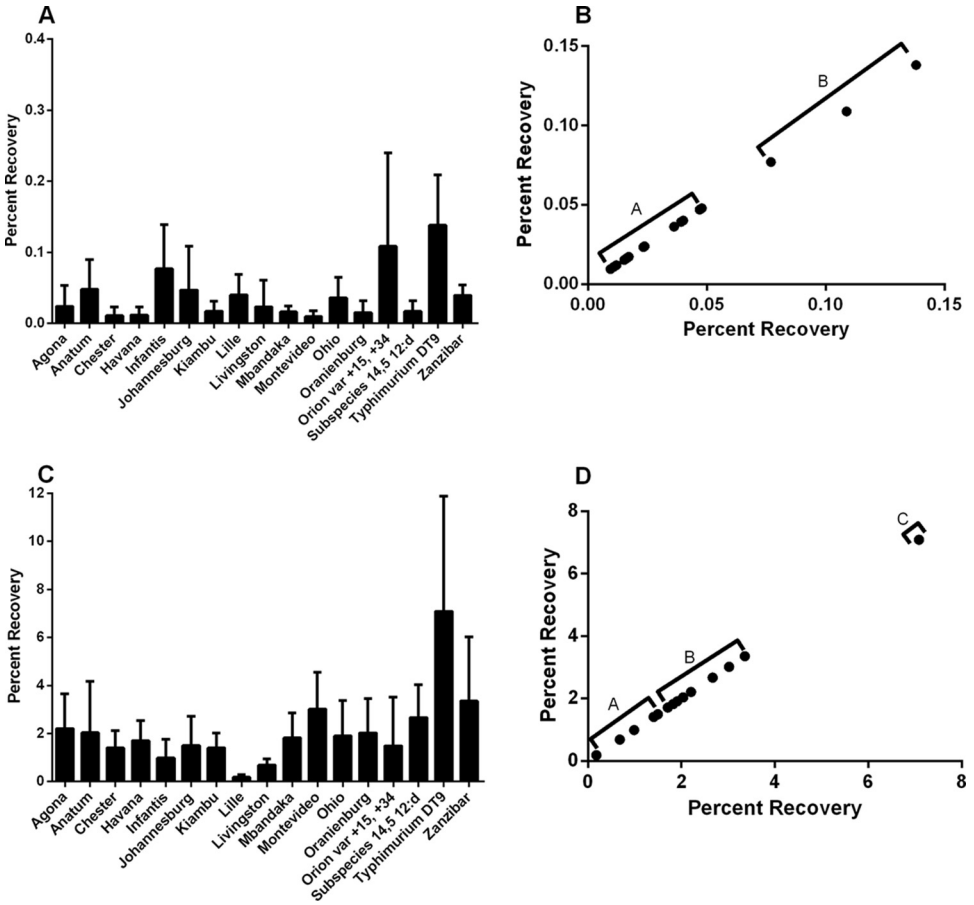

FIG 1 The *in vitro* invasive capacity of 17 nontyphoidal *Salmonella* serovars was assessed using the gentamicin protection assay with the human intestinal epithelial cell line Caco-2. Bacteria were either suspended in normal saline (A) or grown to stationary (liquid) phase in LB broth (C) and then added to cell monolayers at an MOI of 100. Data are presented as mean percent recovery. Assays were repeated five times. Statistical analysis was performed by Kruskal-Wallis ANOVA with *post hoc* analysis utilizing Dunn's multiple-comparison test. A significant effect of serotype for both treatment groups ($P < 0.006$, saline; $P < 0.0002$, LB broth) was detected, but no significant differences were observed between individual serotypes. Cluster analysis (*k*-means) was performed to identify invasion groups. Two invasive types were identified for serovars suspended in normal saline and are identified as group A (low) and group B (moderate) (B). Following growth in LB broth, substantial increases in percent recoveries were observed (C). Three invasion types were identified by cluster analysis and were classified as low (group A), moderate (group B), and high (group C) (D).

ing fimbriae and other virulence genes commonly associated with invasion.

The process of host cell invasion by *Salmonella* spp. has been widely studied using the intestinal epithelial cell line Caco-2 (22). To determine whether egg-associated *Salmonella* serovars have equal invasive potentials following direct exposure from eggshell, bacterial suspensions were prepared in normal saline and subsequently coincubated with cell monolayers for 2 h. *Salmonella* grown on nutrient agar and suspended in normal saline (solid phase) would not have upregulated genes in *Salmonella* pathogenicity island 1, providing a model for direct exposure. Data obtained are represented as percent recovery, determined by the ratio of the amount of bacteria recovered to the initial MOI. Invasive assays were performed in duplicate and repeated five times.

Overall, the invasiveness of the serovars included in this study was low. Despite limited invasion, a significant difference of mean percent recovery between serovars was observed ($P < 0.0066$) (Fig. 1A). A *k*-means cluster analysis revealed two distinct invasive groups (Fig. 1B). The *S. enterica* serovars Agona, Anatum, Chester, Havana, Johannesburg, Kiambu, Lille, Livingston, Mbandaka, Montevideo, Ohio, Oranienburg, subspecies 4,[5],12:d, and Zanzibar had mean percent recoveries of between 0.0095% and 0.047% and clustered in group A (cluster center, 0.03; change about cluster center, 0.016) (Fig. 1A and B). *S.* Typhimurium DT9, *S.* Infantis, and *S. enterica* serovar Orion var. 15^+,34^+ had mean percent recoveries of greater than 0.1% and clustered in group B (cluster center, 0.11; change about cluster mean, 0.03) (Fig. 1A and B).

Environmental conditions are known to influence the expression of *Salmonella* virulence genes (23), in particular, thee genes that regulate the function of flagella and those that are directly involved in cell invasion. To determine how growth conditions affected the invasive capacities, *Salmonella* serovars were cultured to stationary phase (or liquid phase) in LB broth. Consistent with previous reports, we observed substantial increases in invasion. Mean percent recoveries increased from 0.002 to 0.13% (normal saline) (Fig. 1A) to 0.18 to 7.08% (LB broth) (Fig. 1C). *S.* Montevideo was found to have the greatest increase in invasive capacity. Mean percent recovery for *S.* Montevideo suspended in normal saline was 0.009%, which increased over 300-fold to 3.02% fol-

lowing growth in LB broth. *S. enterica* serovars Agona, Chester, Havana, Mbandaka, and Oranienburg as well as subspecies 4,[5],12:d also exhibited substantial increases in invasive capacity, increasing between 90- and 158-fold.

Three distinct invasive groups were identified by *k*-means cluster analysis and were defined as low (group A), moderate (group B), and high (group C) (Fig. 1D). The low-invasive group (cluster center, 1.17; change about cluster mean, 0.99) consisted of *S. enterica* serovars Chester, Havana, Infantis, Johannesburg, Kiambu, Lille, Livingston, and Orion var. 15^+,34^+. Mean percent recoveries obtained for the low-invasive group ranged from 0.18% to 1.7%. *S. enterica* serovars Agona, Anatum, Mbandaka, Montevideo, Ohio, Oranienburg, subspecies 4,[5],12:d, and Zanzibar all clustered into the moderately invasive group (group B) (cluster center, 2.38; change about cluster mean, 0.97) and had mean percent recoveries ranging from 1.83% to 3.36%. *S.* Typhimurium DT9 exhibited the greatest mean percent recovery (7.08%) of the serovars tested. *S.* Typhimurium DT9 clustered by itself in group C (cluster center, 7.08; change about cluster mean, 0.00) and was classified as possessing high invasion capacity.

Invasion experiments using bacteria grown to log phase in LB broth were also performed. No significant difference in invasive capacities was observed for log phase growth compared with bacteria grown to stationary phase in LB broth (data not shown).

Patterson et al. (24) reported upregulation of all genes in *Salmonella* pathogenicity island 1 (SPI-1) of *S.* Typhimurium upon growth in LB broth. Based upon findings of this current work, it was hypothesized that a similar pattern would be observed for other *Salmonella* serovars; further gene expression investigations are, however, necessary. It is important to note that the invasive ability of *Salmonella* Typhimurium DT9 increased from medium to high while other serovars retained their low-invasive status. These results indicate that not all isolates of *Salmonella* recovered from poultry may be equally invasive even after growth to stationary phase (or in liquid phase).

The findings from these intestinal epithelial invasion assays could have significant implications for egg handling as they suggest that some strains of *Salmonella* require favorable growth conditions and environments to stimulate pathogenicity. Moreover, these results also indicate that many *Salmonella* serovars, in particular, the *S.* Typhimurium definitive types, may have a constitutively active virulence gene(s) that enables invasion under any conditions. This may provide a selective advantage for these strains. Future work will be aimed at determining the molecular and cellular mechanisms responsible for these differences.

PCR typing for virulence genes. The *Salmonella* genome possesses many genes whose products are involved in the processes of cellular adhesion and invasion. The 23 genes analyzed in this study were included because they are known to be involved in adhesion, invasion, and survival of *Salmonella* spp. Furthermore, PCR detection of these genes has been widely used as a predictive measure for *Salmonella* virulence (11, 25). Results for virulence gene PCR experiments are summarized in Table 4. Most serovars tested were positive for the majority of the selected virulence genes. *S. enterica* serovars Kiambu, Lille, Livingston, Montevideo, Ohio, and Oranienburg were negative for *avrA*. No amplification of *sopB* was detected for *Salmonella* Lille. *S. enterica* serovars Chester, Havana, Infantis, Johannesburg, Kiambu, Lille, Mbandaka, Montevideo, Oranienburg, and Zanzibar were negative for *sptP*. No PCR amplification was detected for *fliC* except for *S.* Typhimurium DT9. This was also observed for the genes *pefA* and *spvC*. As such, it is likely that PCR results do not indicate that the *Salmonella* serovars tested were negative for these genes but more likely that they possess sufficient genetic variability preventing primer annealing and subsequent amplification. Full-genome sequence analysis studies are warranted to investigate genetic variability.

Comparison of the pathogenicity profiles generated in this study with *in vitro* cell invasion data did not reveal any correlative association between the presence or absence of a gene or genes with virulence. The *Salmonella* genome possesses multiple pathogenicity islands (PIs), which are genetic elements within the bacterial genome that harbor genes associated with virulence. In the species *S. enterica*, 23 *Salmonella* pathogenicity islands (SPIs) have been identified (26) which are considered to play a vital role in the evolution of *Salmonella* (27). Entry of *Salmonella* into intestinal epithelial cells is dependent upon invasion genes that are localized to SPI-1 (28). Bacteria utilize SPI-1 during the gastrointestinal (GI) stage of disease to invade cells and to invoke the inflammatory response (29). For our experiments, different virulence genes for PCR amplification from the *Salmonella* serovars were selected. Our results showed that the majority of the *Salmonella* serovars tested were positive for the virulence genes. Primer sets were designed to the published genome of *Salmonella* Typhimurium LT2. It is known, however, that there is significant genetic variability of virulence gene sequences between *Salmonella* serovars (10). This variability in genomic sequence may translate into variation of function, leading to differences in pathogenicities. However, it is essential to note that possession of a single or a few virulence genes does not endow a strain with pathogenic status unless that strain has acquired the appropriate virulence gene combination to cause disease in a specific host species (30). It should also be noted that there are several other genes which could play important roles in *Salmonella* invasion, and the current study detected the limited set of genes by PCR. Future studies could be directed to investigate virulence gene expression of *Salmonella* serovars at different stages of pathogenesis.

Mouse infection trial with selected isolates. To determine whether *in vitro* cell invasiveness was a correlative measure of *in vivo* pathogenicity, representative *Salmonella* isolates with low (*S.* Infantis and *S.* Lille), medium (*S.* Montevideo and *S.* Oranienburg), and high (*S.* Typhimurium DT9 and ATCC 14028) invasiveness were tested using an *in vivo* mouse challenge model. While the dose rates (10^3 and 10^5 CFU) selected for this study are lower than those used in other reported work, they represent bacterial counts commonly recovered from the egg shell surface (19).

For both the 10^3 and 10^5 doses, mice inoculated with *Salmonella* isolates with high *in vitro* invasiveness (group C) (Table 3) exhibited a higher rate of mortality than those inoculated with isolates with medium (group B) (Table 3) or low (group A) (Table 3) invasiveness (Mantel-Cox log rank test, $P < 0.001$) (Fig. 2A and B.). At the 10^5 dose, the mean survival time for mice inoculated with group C isolates ranged from 5 to 9 days. The majority of mice inoculated with group C isolates died or were euthanized by 9 days p.i.; two animals inoculated with *S.* Typhimurium DT9 and one in the ATCC 14028 inoculation group survived until day 21. No clinical signs of infection or mortality were recorded in mice challenged with group A or B isolates. Similar results were obtained in mice inoculated with 10^3 CFU of *Salmonella* (Fig. 2B). No statistically significant difference was detected in the survival curves between the dose groups ($P > 0.1$). Based on the results of

TABLE 4 Virulence gene profile of *Salmonella* serovars

Virulence gene	*S. enterica* serovar	*avrA*	*hilA*	*invA*	*prgH*	*sipA*	*sipB*	*sipD*	*sopA*	*sopB*	*sptP*	*sitC*	*spiC*	*sifA*	*misL*	*orfL*	*pipD*	*iroN*	*pefA*	*spvC*	*fliC*	*xthA*	*yafD*	*stn*
Agona		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Anatum		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Chester		+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Havana		+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Infantis		+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Johannesburg		+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Kiambu		+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Lille		+	+	−	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Livingston		+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Mbandaka		+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Montevideo		+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Ohio		+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Oranienburg		+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Orion var. 15$^+$,34$^+$		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Subspecies I 4,[5],12:d		+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Typhimurium DT9		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Zanzibar		+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+

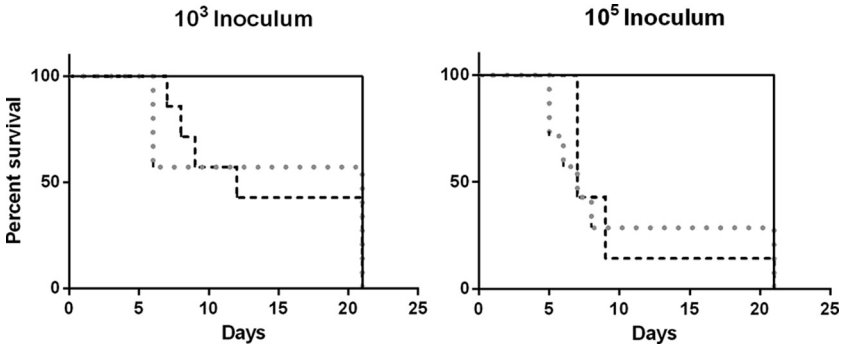

FIG 2 The *in vivo* pathogenicity of *Salmonella* serovars with low (*S.* Infantis and *S.* Lille), moderate (S. Montevideo and *S.* Oranienburg), and high (*S.* Typhimurium DT9 and ATCC 14028) *in vitro* invasiveness was investigated using BALB/c mice. Mice were inoculated with either 10^3 or 10^5 CFU. A Kaplan-Meier survival curve illustrates the percent survival rates of mice challenged with nontyphoidal *Salmonella* isolates. A Mantel-Cox log rank analysis was performed comparing percent survival. Percent survival of mice infected with both *S.* Typhimurium DT9 (gray dotted line) and ATCC 14028 (black dashed line) was significantly less than that of all other treatment groups (black line) at both 10^3 ($P < 0.001$) and 10^5 ($P < 0.0001$) CFU. No significant difference in survival rates was observed between *S.* Typhimurium DT9 and ATCC 14028 for either the low or high dose ($n = 7$ mice per group).

in vitro findings, the virulence of several *Salmonella* serovars was tested in a mouse infection model. The clinical signs of disease and mortality were observed in mice infected with both high and low doses of *S.* Typhimurium DT9 and *S.* Typhimurium ATCC 14028. Mortality was not recorded for infection with medium- or low-invasive *Salmonella* serovars. Lack of morbidity and mortality of *S.* Montevideo-infected mice has been previously reported (31). Similar results were observed for *Salmonella* serovars Infantis, Oranienburg, and Lille, but the mechanisms driving this lack of *in vivo* pathogenicity are not known. These serovars have been previously isolated during food poisoning cases. A single isolate of each serovar was used in this study, and lack of pathogenicity in these serovars could possibly be due to within-serotype genetic variation. Translating the *in vitro* findings into the context of an animal model and subsequently to human disease remains a difficult challenge for any disease process as this introduces a plethora of variables (32).

The TaqMan *Salmonella enterica* detection system does not provide quantification of positive fecal samples. Therefore, to determine the limit of detection of the assay, a standard curve prepared from a known concentration of *Salmonella* spp. (10^8 to 10^0 CFU) was used. The standard curve produced a slope of −3.2, a *y* intercept of 39.4, and an R^2 value of 0.91. A cutoff C_T of 33.7 was used to exclude detection of false positives. A C_T of 33.7 corresponded to 50 CFU of *Salmonella*. Amplification was not recorded in negative-control (LB) samples or any of the treatment groups at day 0 of infection.

In *Salmonella* invasive group A (*Salmonella* serovars Infantis and Lille) infected with a low dose (10^3 CFU), both qPCR and culture tests indicated that the shedding was detected until days 9 and 15 p.i., respectively. Highest shedding (4.5 log CFU) was recorded on day 6 p.i. in the *S.* Infantis-infected group (Table 5). *Salmonella* was isolated up to 18 days p.i. after a higher dose (10^5 CFU) of infection with *S.* Infantis and *S.* Lille. On other hand, *Salmonella* shedding was detected by qPCR up to 15 and 9 days p.i. after the higher dose (10^5 CFU) of infection with *S.* Infantis and *S.* Lille, respectively. For the *S.* Infantis and *S.* Lille infection group, there was significant difference in *Salmonella* shedding between time points (days p.i.) ($P < 0.0001$). There was significant interaction between dose and days p.i. ($P < 0.0001$). There was a significant difference in *Salmonella* shedding between the high and low doses for the *S.* Infantis group ($P < 0.0001$). qPCR results showed that in *Salmonella* invasion group B (*Salmonella* serovars Oranienburg and Montevideo), *Salmonella* shedding was recorded until day 18 p.i. in mice infected with both high and low doses of *S.* Montevideo. However, *S.* Oranienburg was isolated from feces until 15 days p.i. in mice infected with a low dose (Table 5). For the *S.* Oranienburg and *S.* Montevideo groups, there was a significant difference in *Salmonella* shedding between doses and time points (days p.i.) (for both parameters, $P < 0.0001$). There was significant interaction between dose and days p.i. ($P < 0.0001$). In *Salmonella* invasion group C infected with a low dose (*S.* Typhimurium DT9), *Salmonella* shedding was recorded until day 18 pi. by both qPCR and the culture method. In the high-dose infected group, *Salmonella* was detected until day 18 p.i. by the qPCR method while *Salmonella* could be isolated until day 9 p.i. by the culture method. There was no significant difference in shedding results between infection doses; however, a significant difference was recorded for days p.i. ($P < 0.0001$).

Some disparity was observed between *Salmonella* culture and qPCR results. Using the culture method, *Salmonella* was isolated from qPCR-negative samples. The culture method used in this study enabled the isolation of *Salmonella* at levels below the detection limit established for the qPCR. Some culture-negative samples, however, tested positive by qPCR (*S.* Typhimurium DT9, *S.* Infantis, and *S.* Montevideo). It is essential to note that qPCR methods detect both viable and nonviable bacteria while culture methods detect only viable bacteria. Such disparity in culture versus qPCR results has also been observed during *Salmonella* detection/isolation from swine feces (33).

Persistent shedding of *Salmonella* in feces contributes to the transmission of the bacteria to naive hosts. Variable shedding patterns were observed among the six serovars included in this study. Similar patterns have been shown for other nontyphoidal *Salmonella* serovars (31). Genes carried on SPI-2 are known to contribute toward the maintenance of long-term infection (34). It is likely, therefore, that genetic variation within these genes may contribute to the ability of some serovars to establish a persistent infection.

Conclusion. In conclusion, this study has shown that although eggs or egg products are frequently implicated in *Salmonella* infection in humans, not all *Salmonella* isolates obtained from egg or

egg-associated products are equally invasive and/or pathogenic, as measured by invasion assays using human intestinal epithelial cells (Caco-2) and live BALB/c mice. Further investigations are necessary to study the comparatively high- and low-virulence *Salmonella* genomes and their detailed molecular mechanisms in order to understand the pathogenesis.

It is also important that only one isolate per serovar was used in this study. Within-serotype variation in *Salmonella* virulence has been demonstrated for several serotypes (17). Although the current study has characterized the pathogenicity of an individual serovar, future studies need to be directed toward investigation of within-serotype variation. Collectively, our data demonstrated a presence of virulence genes from SPI-1 to SPI-4 in high-, medium-, and low-invasive *Salmonella* serovars. Our data also greatly expand on the known significant strain-to-strain variation in *in vitro* and *in vivo* pathogenicity and highlight the need for further comparative genomic and phenotypic studies.

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TABLE 5 qPCR and culture detection of *Salmonella* from mice inoculated with 10^3 and 10^5 CFU

Salmonella serovar	Dose	Detection by day p.i. and method[a]												*P* value for qPCR test[b]		
		0		3		6		9		12		15		18		
		qPCR	Culture	qPCR	Culture	qPCR	Culture	qPCR	Culture	qPCR	Culture	qPCR	Culture	qPCR	Culture	Dose	Day p.i.	Dose × days p.i.
Typhimurium DT 9	10^3	ND	−	3.8 ± 0.13	−	3.6 ± 0.09	+	ND	−	3.1 ± 0.18	+	3.0 ± 0.1	+	3.0 ± 0.1	+	NS	<0.0001	<0.0001
	10^5	ND	−	3.7 ± 0.01	−	2.8 ± 0.02	+	4.0 ± 0.01	+	2.3 ± 0.2	−	ND	−	3.6 ± 0.09	−			
Infantis	10^3	ND	−	3.0 ± 0.02	+	4.6 ± 0.02	+	3.1 ± 0.07	+	ND	−	ND	−	ND	−	<0.0001	<0.0001	<0.0001
	10^5	ND	−	0	+	0	+	3.0 ± 0.13	+	ND	+	2.7 ± 0.04	+	ND	+			
Lille	10^3	ND	−	3.0 ± 0.05	+	1.5 ± 0.2	+	ND	+	ND	−	2.9 ± 0.08	+	ND	−	NS	<0.0001	<0.0001
	10^5	ND	−	3.1 ± 0.07	+	2.9 ± 0.11	+	3.0 ± 0.01	−	ND	−	ND	+	ND	+			
Oranienburg	10^3	ND	−	3.0 ± 0.33	+	ND	−	ND	+	ND	−	ND	+	ND	−	<0.0001	<0.0001	<0.0001
	10^5	ND	−	3.7 ± 0.04	+	3.1 ± 0.16	+	3.0 ± 0.15	−	2.8 ± 0.07	+	3.2 ± 0.01	+	ND	+			
Montevideo	10^3	ND	−	3.6 ± 0.06	+	2.9 ± 0.01	+	2.8 ± 0.2	+	2.7 ± 0.03	−	2.8 ± 0.04	+	3.0 ± 0.06	+	<0.0001	<0.0001	0.0004
	10^5	ND	−	3.2 ± 0.11	+	3.5 ± 0.06	+	3.4 ± 0.03	+	3.5 ± 0.33	+	3.6 ± 0.01	+	4.1 ± 0.02	+			
Typhimurium ATCC 14028	10^3	ND	−	3.3 ± 0.08	+	3.5 ± 0.18	+	4.1 ± 0.23	+	4.1 ± 0.94	+	3.9 ± 0.25	+	3.1 ± 0.72	+	NS	0.0001	NS
	10^5	ND	−	4.1 ± 0.08	+	3.1 ± 0.32	+	1.6 ± 0.6	+	2.9 ± 0.45	+	3.2 ± 0.24	+	3.7 ± 0.06	+			

[a] Cultures were either positive (+) or negative (−). All qPCR values presented are in CFU/g of feces ± standard error of the mean. ND, none detected; values below the qPCR limit of detection.

[b] The *P* values indicate the significance of the indicated parameter. NS, not significant; Dose × days p.i., interaction of dose and days p.i.

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